REPRESSING GENE EXPRESSION IN PLANTS

The present invention relates to repression of gene expression. More specifically the invention relates to repression of gene expression in plants by histone deacetylase, and histone deacetylase enzyme homologs.

BACKGROUND OF THE INVENTION

Posttranslational modifications of histones in chromatin are important mechanisms in the regulation of gene expression. Acetylation of core histones is correlated with transcriptionally active chromatin of eukaryotic cells. Acetylation is thought to weaken the interactions of histones with DNA and induce alterations in nucleosome structure. These alterations enhance the accessibility of promoters to components of the transcription machinery, and increase transcription.

Histone deacetylation is thought to lead to a less accessible chromatin conformation, resulting in the repression of transcription (e.g. Pazin and Kadonaga, 1997; Struhl, 1998). The role of the yeast histone deacetylase, RPD3, in transcriptional repression was first discovered through a genetic screen for transcriptional repressors in *S. cerevisiae* (Vidal and Gaber, 1991). Since then, a number of yeast and mammalian histone deacetylase genes have been cloned (Rundlett et al., 1996; Emiliani et al., 1998; Hassig et al., 1998; Verdel and Khochbin, 1999). Most eukaryotic histone deacetylases show some sequence homology to yeast *RPD3*, suggesting that these proteins are all members derived from a single gene family (Khochbin and Wolffe, 1997; Verdel and Khochbin, 1999). In yeast and mammalian cells, the RPD3 histone deacetylases mediate transcriptional repression by interacting with specific DNA-binding proteins or associated corepressors and by recruitment to target promoters (Alland et al., 1997; Kadosh and Struhl, 1997; Hassig et al., 1997; Nagy et al., 1997; Gelmetti et al., 1998). Recently, a second family of histone deacetylases, HDA1 and related proteins, were identified in yeast and mammalian cells (Rundlett et al., 1996; Fischle et al., 1999; Verdel and Khochbin, 1999). The deacetylase domain of HDA1-related proteins is homologous to but significantly different from that

of *RPD3* (Fischle et al., 1999; Verdel and Khochbin, 1999). These proteins also appear to be functionally different from RPD-like proteins in yeast cells (Rundlett et al., 1996). WO 97/35990 discloses mammalian-derived histone deacetylase (HDx) gene sequences, gene products, and uses for these sequences and products. There is no disclosure of the use of these gene products for repressing gene expression.

In plants, an *RPD3* homolog was first discovered in maize and it complemented the phenotype of a *rpd3* null mutant of the yeast *S. cerevisiae* (Rossi et al, 1998). HD2 was also identified from maize that shows no sequence homology to yeast RPD3 or RPD3-related proteins (Lusser et al., 1997).

Even though histone deacetylation is thought to lead to repression of transcription, this has never been tested in plant systems. WO 98/48825 discloses the use of histone deacetylase (HD) for repressing gene expression in mammalian cell culture, however, the use of HD, or modified HD in plant gene repression is not disclosed. There is a plethora of information relating to the up-regulation of gene expression in plants, however, little is known on systems that can down regulate gene-expression. Thus, there is a need to develop regulatory systems for selectively repressing gene expression in plants.

The present invention pertains to novel histone deacetylase enzymes obtained from a plant. Four novel genes encoding histone deacetylases (*AtRPD3A*, *AtRPD3B*, *AtHD2A* and *AtHD2B*) and fragments thereof, were shown to be involved in the regulation of gene transcription within plants.

It is an object of the invention to overcome disadvantages of the prior art.

The above object is met by the combination of features of the main claims, the sub-claims disclose further advantageous embodiments of the invention.

SUMMARY OF THE INVENTION

The present invention relates to repression of gene expression by histone deacetylase enzymes. More specifically the invention relates to repression of gene expression in plants by histone deacetylase enzymes.

According to the present invention there is provided a method of regulating gene expression in a transgenic plant comprising, introducing into a plant:

i) a first chimeric nucleotide sequence comprising a first regulatory element in operative association with a gene of interest, and a controlling sequence: and

ii) a second chimeric nucleotide sequence comprising a second regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase and a nucleotide sequence encoding a DNA binding protein, the DNA binding protein having an affinity for the controlling sequence,

to produce the transgenic plant, and growing the transgenic plant.

The present invention is directed to the above method wherein the step of introducing comprises transforming the plant with the first, and the second, chimeric nucleotide sequence. Furthermore, the step of introducing comprises transforming a first plant with the first chimeric nucleotide sequence, and transforming a second plant with the second chimeric nucleotide sequence, followed by a step of crossing the first and the second plant, to produce the transgenic plant. Also included is the above method, wherein the step of introducing comprises transforming a plant with the first chimeric nucleotide sequence, followed by transforming the same plant with the second chimeric nucleotide sequence, or co-transforming a plant with both the first and second chimeric nucleotide sequences.

The present invention embraces the method as described above wherein the histone deacetylase, within the step of introducing, is selected from the group consisting of *AtRPD3A*, *AtRPD3B*, *AtHD2A AtHD2B*, an analogue, fragment, or derivative of *AtRPD3A*, *AtRPD3B*, *AtHD2A AtHD2B*, and a nucleotide sequence that hybridizes to *AtRPD3A*, *AtRPD3B*, *AtHD2A AtHD2B* at 65^{0}C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS,

and 1 mM EDTA, wherein the analog, fragment, derivative, or nucleotide sequence that hybridizes encodes a product that exhibits repression of gene expression activity.

The present invention also relates to the method as described above wherein the upstream activating sequence and the DNA binding protein, within the step of introducing, are a Gal4 upstream activating sequence and a GAL4-binding protein, respectively. Furthermore, the first and the second regulatory region are selected from the group consisting of constitutive, tissue specific, developmentally-regulated, and inducible regulatory elements.

This invention is also directed to an isolated nucleotide sequence, selected from the group consisting of:

i) SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7;

ii) an analog, derivative, fragment of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7; and

iii) a nucleotide sequence that hybridizes to SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7 at 65°C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1 mM EDTA,

wherein the analog, derivative, fragment or the nucleotide sequence that hybridizes encodes a product that exhibits repression of gene expression activity. Furthermore, according to the present invention, there is also provided a chimeric construct comprising a regulatory element in operative association with the isolated nucleotide sequence as defined above, as well as a vector comprising the chimeric construct.

The present invention also pertains to an isolated amino acid sequence, selected from the group consisting of:

i) SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8; and

ii) an analog, derivative, fragment of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8,

wherein the analog, derivative, or fragment exhibits repression of gene expression activity.

The present invention includes a transgenic plant, a transgenic plant cell, a transgenic seed, comprising said isolated nucleotide sequence as defined above.

The present invention is directed to a method of regulating gene expression in a plant comprising,

i) introducing into the plant a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase and a nucleotide sequence encoding a DNA binding protein, to produce a transgenic plant; and

ii) growing the transgenic plant,

wherein the DNA binding protein has an affinity for a native controlling sequence within the plant.

The present invention also provides a method for altering a biochemical, physiological or developmental pathway of an organism comprising:

i) introducing into an organism a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase and a nucleotide sequence encoding a DNA binding protein specific for a controlling sequence; and

ii) growing the organism.

The present invention includes a method for identifying a DNA binding protein comprising:

i) introducing into a plant a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase fused with a nucleotide sequence of interest and of unknown function, to produce a transgenic plant;

ii) growing the transgenic plant; and

iii) examining the transgenic plant to determine whether the chimeric nucleotide sequence, comprising the nucleotide sequence of interest has an effect on plant phenotype.

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a sub-combination of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

FIGURE 1 shows the nucleotide and predicted amino acid sequences of several HD's of the present invention. Figure 1 (A) shows the nucleotide and amino acid of *AtRPD3A* (SEQ ID NO's:1 and 2, respectively). Figure 1 (B) shows the nucleotide and amino acid sequence of *AtRPD3B* (SEQ ID NO's:3 and 4, respectively).

FIGURE 2 shows nucleotide and predicted amino acid sequences of several more HD's of the present invention. Figure 2 (A) shows the nucleotide and amino acid of *AtHD2A* (SEQ ID NO's:5 and 6, respectively. Figure 2 (B) shows the nucleotide and amino acid of AtHD2B (SEQ ID NO': 7 and 8, respectively).

FIGURE 3 displays the amino acid sequence alignment of the *AtRPD3A*, *AtRPD3B*, maize RPD3 (ZmRPD3) and yeast RPD3. Identical amino acids are shaded in black. The amino acids with asterisks represent residues with potential roles in deacetylase activity.

FIGURE 4 displays the amino acid sequence alignment of *AtHD2A*, *AtHD2B* and maize HD2 (ZmHD2). Identical amino acids are shaded in black. The amino acids with asterisks are the predicted histone deacetylase catalytic residues. The extended acidic domains are underlined.

FIGURE 5 shows a Genomic Southern blot analysis of *AtRPD3A* and *AtRPD3B*. *Arabidopsis* genomic DNA (approximately 10 μg) was digested with *EcoR*I , *Hind*III, *Pst*I, or *Xho*I, fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled *AtRPD3A* (A) and *AtRPD3B* (B) cDNA probes.

FIGURE 6 shows a Genomic Southern blot analysis of *AtHD2A* and *AtHD2B*. *Arabidopsis* genomic DNA (approximately 10 μg) was digested with *EcoR*I (lane 1), *Hind*III (lane 2), *Pst*I (lane 3), or *Xho*I (lane 4), fractionated by agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled *AtHD2A* (A) and *AtHD2B* (B) cDNA probes.

FIGURE 7 displays a Northern blot analysis of the *AtRPD3A* transcripts. Total RNA was isolated from leaves (L), stems (S), flowers and young siliques (F), and whole plants (W) of *Arabidopsis thaliana*. Five microgram of total RNA was probed with *AtRPD3A* and a loading control probe (actin).

FIGURE 8 displays a Northern blot analysis of the *AtHD2A* and *AtHD2B* transcripts. Total RNA was isolated from leaves (L), stems (S), flowers and young siliques (F), and whole plants (W) of *Arabidopsis thaliana*. Five microgram of total RNA was probed with *AtHD2A*, *AtHD2B* and a loading control probe (actin).

FIGURE 9 shows a schematic of the effector and reporter plasmids comprising *AtRPD3A*, and the repression of the UAS_{GAL4}-BtCUP-GUS fusion gene by *AtRPD3A* protein in transient expression assays. Figure 9 (A) shows a schematic diagram of the effector and reporter constructs used in co-bombardment experiments. The effector constructs contained the tCUP promoter fused to the *AtRPD3A* coding region which was fused to the DNA binding domain of GAL4 (GAL4BD) and the polyadenylation signal of nopaline synthetase gene (Nos-T). The reporter construct (UAS_{GAL4}-BtCUP-GUS) contained the upstream activating sequence of GAL4 protein tandemly repeated two times (UAS_{GAL4}) fused to the -394tCUP promoter-GUS construct. Figure 9 (B) shows repression of the UAS_{GAL4}-BtCUP-GUS fusion gene by *AtRPD3A* protein. The reporter gene was co-bombarded with each effector plasmid or a control plasmid pUC19 as a control treatment. GUS activity was reported as picomoles of 4-methylumbelliferone per milligram of protein per minute. Bars indicate the standard error of three replicates.

FIGURE 10 shows the repression of the UAS_{GAL4}-BtCUP-GUS fusion gene by *AtHD2A* protein in transient expression assays. Figure 10 (A) shows a schematic diagram of the effector and reporter constructs used in cobombardment experiments. The reporter construct contains the upstream activating sequence of GAL4 tandem repeated two times (UAS_{GAL4} x 2) and fused to the -394-tCUP promoter-GUS construct. The effector constructs contain the GAL4 DNA binding domain (amino acids 1-147) fused to the full-length *AtHD2A* (*AtHD2A*, 1-245) or a series deletions of *AtHD2A*. HDAC refers to the predicted histone deacetylase catalytic domain. Acidic R refers to the extended acidic amino acid domain and C2H2 refers to the putative zinc finger. Figure 10 (B) shows repression of the UAS_{GAL4}-BtCUP-GUS fusion gene by *AtHD2A* and its deletions. The reporter gene was cobombarded with each effector plasmid, or control plasmid pUS19 as control treatments. GUS activity was reported as picomoles of 4-methylumbelliferone per milligram of protein per minute. Bars indicate the standard error of three replicates.

FIGURE 11 displays the structure of the plasmids used for *Arabidopsis* transformation of AtRPD3A. Figure 11 (A) shows full-length *AtRPD3A* cDNA in which the positions of *Sac*I and *Ssp*I restriction sites are indicated. Figure 11 (B) shows antisense construct in which the 519 bp fragment of the truncated *AtRPD3A* cDNA in an antisense orientation was driven by the -394-tCUP promoter. Nos-T refers to the polyadenylation signal of the nopaline synthetase gene.

FIGURE 12 shows the Northern analysis of *AtRPD3A* mRNA in transgenic plants. Analysis of *AtRPD3A* expression in wild-type line (WT) and antisense lines (B2, B5 and A1). Five microgram of total RNA isolated from leaves was probed with *AtRPD3A* cDNA probe and a loading control probe (actin).

FIGURE 13 shows the Northern analysis of *AtHD2A* antisense plants. Analysis of endogenous *AtRHD2A* expression of wild-type line (WT) and antisense transgenic

lines (1-5). Five microgram of total RNA isolated from flower and young siliques was probed with an endogenous *AtRPD3A* specific probe and an antisense *AtRPD3A* specific probe.

FIGURE 14 displays the phenotypic abnormalities of plants expressing *AtRPD3A* antisense RNA. Wild-type plant (A) and antisense *AtRPD3A* transgenic plants (B and C) were grown for 6 weeks. The transgenic plants show a delay in flowering compared to the wild-type plant.

FIGURE 15 displays the phenotype of *AtHD2A* antisense plants. An *AtHD2A* antisense plant is semi-sterile and exhibits a reduced seed set. The insert shows a wild-type stem with full silique elongation (left) and a stem from an *AtHD2A* antisense plant with stunted siliques (right).

FIGURE 16 displays scanning electron micrographs of siliques. Figure 16 (A) shows siliques formed on the wild-type plant. Figure 16 (B) shows siliques formed on an antisense *AtHD2A* transgenic plant.

FIGURE 17 shows an outline of an experiment to demonstrate repression of expression of a gene in a tissue-specific manner. Figure 17(A) outlines a binary transrepression system involving the use of a tissue-specific regulatory element and constructs shown in Figure 17 (B). A reporter gene (expression construct) under the control of a constitutive promoter is active when introduced into a reporter plant. Effector genes, under control of tissue specific regulatory regions are introduced into effector plants. Transgene repression is achieved by crossing reporter plant lines with effector lines that express a repressor (eg. histone deacetylase), and a controlling sequence binding domain that specifically recognizes a control sequences of the reporter gene. The pattern of reporter gene repression will reflect the pattern of repressor expression, allowing a gene of interested to be repressed under a variety of regimes by crossing to an appropriate effector line. The upper lob of the schematic plant represents the fruiting body of the plant, for example the seeds, while the horizontal lobes represent

leaves. Black areas represent tissues exhibiting reporter gene expression, while grey and white areas represent no reporter gene expression. Grey regions indicate expression of the HD/CS-BD (effector) constructs. for example, either NAP1-GAL4/HD, or tCUP-GAl4/HD. Figure 17 (B) shows a schematic of the plasmids used for repressing transgene expression in transgenic plants. The effector constructs contained the tCUP promoter (Effector 1) or napin promoter (NAP; Effector 2)) fused to the fusion of the GAL4BD with the *AtHD2A* coding region and the polyadenylation signal of nopaline synthetase gene (Nos-T). The reporter constructs ($GAL4_{UAS}$-tCUP-GUS and $GAL4_{UAS}$-35S-GUS) contained the upstream activating sequence of GAL4 protein tandem repeated two times ($GAL4_{UAS}$) fused to the -394-tCUP or 35S promoter-GUS constructs.

FIGURE 18 shows reporter gene activity within plants produced following the experiment outlined in Figure 17. Figure 18 (A) Histochemical analysis of GUS expression in the seedlings, flowers and seeds of the reporter line UAS_{GAL4}-tCUP-GUS. Figure 18 (B). GUS expression is repressed in the seedlings, flowers and seeds of the crossing tCUP-GAL4/AtHD2A X UAS_{GAL4}-tCUP-GUS F1 progeny. Figure 18 (C). GUS expression is specifically repressed in the seeds of the crossing NAP1-GAL4/AtHD2A X UAS_{GAL4}-tCUP-GUS F1 progeny.

FIGURE 19 shows GUS expression in the plants following crossing reporter lines and effector lines progeny as outlined in Figure 17, or sequential transformation of plants with reporter and effector constructs. Figure 19 (A) shows GUS specific activity analyzed in the leaves and seeds of the reporter line tCUP-GUS and F1 progeny produced from the cross between tCUP-GUS **X** 35S-GAL4/AtHD2A, and tCUP-GUS **X** NAP1-GAL4/AtHD2A. Ten to 15 plants from the reporter line and each cross were analyzed. Figure 19 (B) shows GUS specific activity analyzed in leaves of plants transformed with tCUP-GUS (UAS_{GAL4}-tCUP-GUS) (control 1, 2), and plants sequentially transformed with tCUP-GUS (UAS_{GAL4}-tCUP-GUS) followed by a second transformation with either 35S-GAL4/AtHD2A (35S-GAL4/HD:tCUP-GUS) or Napin-GAL4/AtHD2A (NAP1-GAL4/HD:tCUP-GUS). Control 3, plants transformed with 35S-GAL4/AtHD2A only. Three plants were assayed for each

treatment. Figure 19 (C) shows GUS specific activity analyzed in seeds of plants transformed with tCUP-GUS (UAS_{GAL4}-tCUP-GUS) (control 1, 2), and plants sequentially transformed with tCUP-GUS (UAS_{GAL4}-tCUP-GUS) followed by a second transformation with either 35S-GAL4/AtHD2A (35S-GAL4/HD:tCUP-GUS) or Napin-GAL4/AtHD2A (NAP1-GAL4/HD:tCUP-GUS). Control 3, plants transformed with 35S-GAL4/AtHD2A only. Three plants were assayed for each treatment.

FIGURE 20 shows activation of the GCC box-mediated transcription of GUS reporter gene by Pti4 protein in transient expression assays. Figure 20 (A) outlines a schematic diagram of the effector and reporter constructs used in co-bombardment experiments. The reporter construct contains two GCC-boxes fused to the -62tCUP minimal promoter-GUS construct. The effector constructs contain the *Pti4* cDNA fused to the *Nos* terminator driven by the 35S or tCUP promoter. Figure 20(B) shows activation of the GCC/GUS fusion gene by Pti4. The reporter plasmid, GCC/GUS, was co-bombarded with each effector plasmid or the control plasmid pUC19. GUS activity was reported as picomoles of 4-methylumbelliferone per milligram of protein per minute. Bars indicate the standard error of three replicates.

FIGURE 21 shows Northern blot analysis of the *Pti4* transgenic plants. Total RNA was isolated from wild-type (WT) and transgenic lines (1-6). Lanes 1 to 6 correspond to transgenic lines tCUP/Pti4-1, tCUP/Pti4-3, tCUP/Pti4-4, tCUP/Pti4-5, 35S/Pti4-1, and 35S/Pti4-2, respectively. Five micrograms of total RNA were probed with a *Pti4* cDNA probe and a basic chitinase (BC) probe. Photographs of the 25S rRNA bands on the ethidium-bromide-stained gel are shown as a measure of approximately equal loading of the gels.

FIGURE 22 shows length of hypocotyl of transgenic *Arabidopsis* seedlings. Surface-sterilized seeds from wild-type (WT) and transgenic lines (tCUP/Pti4-3 and tCUP/Pti4-1) were planted in growth medium and cold treated at 4°C for 4 days before germination and growth in the dark at 23°C for 72 hr in the presence (with

ACC) or absence (without ACC) of 1-aminocyclopropane-1-carboxylic acid. The lengths of seedling hypocotyls were measured to the closest millimeter. 14 to 20 seedlings from each line were measured. Error bars correspond to the standard error.

FIGURE 23 Phenotype of *Pti4* overexpression in transgenic seedlings. Each panel is composed of two etiolated *Arabidopsis* seedling. Surface-sterilized seeds were planted in growth medium and cold treated at 4°C for 4 days before germination and growth in the dark at 23°C for 72 hr. Figure 23 (A) Wild-type incubated without aminocyclopropane carboxylic acid (ACC); Figure 23 (B) Wild-type displaying the triple response in the presence of 10 μM ACC; Figure 23 (C) tCUP/Pti4-3 transgenic seedlings incubated without ACC; Figure 23 (D) tCUP/Pti4-3 transgenic seedlings incubated in the presence of 10 μM ACC.

FIGURE 24 shows the phenotype of *Pti4-AtHD2A* overexpression in transgenic seedlings. Each panel is composed of two etiolated *Arabidopsis* seedling. Surface-sterilized seeds were planted in growth medium and cold treated at 4°C for 4 days before germination and growth in the dark at 23°C for 72 hr. Figure 24 (A) shows the wild-type. Figure 24 (B) shows tCUP/Pti4-3 plants. Figure 24 (C) shows tCUP/Pti4-AtHD2A seedlings. The photos were taken after plants were grown for 5 weeks in a growth chamber (16 hr of light and 8 hr of darkness at 23°C).

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to repression of gene expression by histone deacetylase enzymes. More specifically the invention relates to repression of gene expression in plants by histone deacetylase enzymes, and histone deacetylase enzyme homologs.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

Histone deacetylases (HD) can be grouped into three families: (1) *S. cerevisiae* RPD3 and RPD3-related proteins (Rundlett et al., 1996; Emilliani et al., 1998); (2) *S. cerevisiae* HDA1 and related proteins (Fischle et al., 1999; Verdel and Khochibin, 1999) and; (3) Zea mays HD2 and related proteins (Lusser et al., 1997). Sequence analysis performed on these proteins showed that most of them display conserved features. For example, RPD3 related proteins maintain a highly-homologous N-terminal domain and a more variable short C-terminal region (Khochbin and Wolffe, 1997). The RPD3 homology domain is also shared by several prokaryotic proteins interacting with various acetylated substrates (Leipe and Landsman, 1997; Ladomery et al.,1997). Mutagenesis analysis of this homology domain confirmed that this domain is inextricably linked to its deacetylase enzymatic activity (Hassig et al., 1998; Kadosh and Struhl, 1998). The second family of histone deacetylases, comprising HDA1 and related-proteins also maintain a highly homologous deacetylase domain, but the domain is significantly different from that of RPD3 and RPD3-like proteins. Similarly, HD2 and HD2-like proteins show no sequence homology to RPD3-like and HDA1-like families of histone deacetylases.

As described in more detail below, a plant EST database was screened using yeast *RPD3* or maize *HD2*. Two EST clones were identified corresponding to the yeast *RPD3* sequence and two clones were identified corresponding to the maize *HD2* sequence (Figure 1 and 2). These clones were termed *AtRPD3A* (SEQ ID NO:1), *AtRPD3B* (SEQ ID NO:2), *AtHD2A* (SEQ ID NO:3) and *AtHD2B* (SEQ ID NO:4), respectively.

The expression of *AtRPD3A and AtRPD3B* transcripts, determined by Northern hybridization (Figure 7 and 8) revealed that *AtRPD3A* RNA accumulated to relatively high levels in the leaves, stems, flowers and young siliques. The pattern of *AtHD2A* and *AtHD2B* RNA expression indicated that *AtHD2A* RNA accumulated in the flowers and young siliques, while *AtHD2B* RNA, accumulated the stem, flowers and young siliques and to a somewhat lower level in the leaves.

The HD's of the present invention, and those of the prior art, may be used to repress the expression of a gene of interest within a plant by targeting a desired HD to a nucleotide sequence containing the gene of interest. While not wishing to be bound by theory, the repression of gene expression activity via locally altering chromatin structure is made possible by targeting a HD to a nucleotide sequence within the vicinity of a gene of interest. The localized deacetylation of histones may result in the observed repression of transcription as described herein. By "histone deacetylase" (HD) it is meant any HD as known within the art. These include the HD's as described of the present invention as well as other plant, animal or microbial HD's. Furthermore, by "repression of gene expression activity" it is meant the reduction in the level of mRNA, protein, or both mRNA and protein, encoded by the gene of interest. Repression of gene expression activity may result from the down regulation of transcription.

By "regulatory region" or "regulatory element" it is meant a portion of nucleic acid typically, but not always, upstream of a gene, which may be comprised of either DNA or RNA, or both DNA and RNA. A regulatory element may be capable of mediating organ specificity, or controlling developmental or temporal gene activation. A "regulatory element" includes promoter elements, basal (core) promoter elements, elements that are inducible in response to an external stimulus, elements that mediate promoter activity such as negative regulatory elements or transcriptional enhancers. "Regulatory element", as used herein, also includes elements that are active following transcription, for example, regulatory elements that modulate gene expression such as translational and transcriptional enhancers, translational and transcriptional repressors, upstream activating sequences, and mRNA instability determinants. Several of these

latter elements may be located proximal to the coding region. In the context of this disclosure, the term "regulatory element" or "regulatory region" typically refers to a sequence of DNA, usually, but not always, upstream (5') to the coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at a particular site. However, it is to be understood that other nucleotide sequences, located within introns, or 3' of the sequence may also contribute to the regulation of expression of a coding region of interest. An example of a regulatory element that provides for the recognition for RNA polymerase or other transcriptional factors to ensure initiation at a particular site is a promoter element. A promoter element comprises a basal promoter element, responsible for the initiation of transcription, as well as other regulatory elements (as listed above) that modify gene expression.

There are several types of regulatory elements, including those that are developmentally regulated, inducible and constitutive. A regulatory element that is developmentally regulated, or controls the differential expression of a gene under its control, is activated within certain organs or tissues of an organ at specific times during the development of that organ or tissue. However, some regulatory elements that are developmentally regulated may preferentially be active within certain organs or tissues at specific developmental stages, they may also be active in a developmentally regulated manner, or at a basal level in other organs or tissues within the plant as well.

An inducible regulatory element is one that is capable of directly or indirectly activating transcription of one or more DNA sequences or genes in response to an inducer. In the absence of an inducer the DNA sequences or genes will not be transcribed. Typically the protein factor, that binds specifically to an inducible regulatory element to activate transcription, may be present in an inactive form which is then directly or indirectly converted to the active form by the inducer. However, the protein factor may also be absent. The inducer can be a chemical agent such as a protein, metabolite, growth regulator, herbicide or phenolic compound or a physiological stress imposed directly by heat, cold, salt, or toxic elements or indirectly through the action of a pathogen or disease agent such as a virus. A plant cell containing an inducible regulatory element may be

exposed to an inducer by externally applying the inducer to the cell or plant such as by spraying, watering, heating or similar methods. Inducible elements may be derived from either plant or non-plant genes (e.g. Gatz, C. and Lenk, I.R.P.,1998, Trends Plant Sci. 3, 352-358; which is incorporated by reference). Examples, of potential inducible promoters include, but not limited to, teracycline-inducible promoter (Gatz, C.,1997, Ann. Rev. Plant Physiol. Plant Mol. Biol. 48, 89-108; which is incorporated by reference), steroid inducible promoter (Aoyama, T. and Chua, N.H.,1997, Plant J. 2, 397-404; which is incorporated by reference) and ethanol-inducible promoter (Salter, M.G., et al, 1998, Plant Journal 16, 127-132; Caddick, M.X., et al,1998, Nature Biotech. 16, 177-180, which are incorporated by reference) cytokinin inducible *IB6* and *CKI1* genes (Brandstatter, I. and Kieber, J.J.,1998, Plant Cell 10, 1009-1019; Kakimoto, T., 1996, Science 274, 982-985; which are incorporated by reference) and the auxin inducible element, DR5 (Ulmasov, T., et al., 1997, Plant Cell 9, 1963-1971; which is incorporated by reference).

A constitutive regulatory element directs the expression of a gene throughout the various parts of a plant and continuously throughout plant development. Examples of known constitutive regulatory elements include promoters associated with the CaMV 35S transcript. (Odell et al., 1985, *Nature*, **313**: 810-812), the rice actin 1 (Zhang et al, 1991, *Plant Cell*, **3**: 1155-1165) and triosephosphate isomerase 1 (Xu et al, 1994, *Plant Physiol.* **106**: 459-467) genes, the maize ubiquitin 1 gene (Cornejo et al, 1993, *Plant Mol. Biol.* **29**: 637-646), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et al, 1995, *Plant Mol. Biol.* **29**: 637-646), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995 *Plant Mol. Biol.* **29**: 995-1004). The term "constitutive" as used herein does not necessarily indicate that a gene under control of the constitutive regulatory element is expressed at the same level in all cell types, but that the gene is expressed in a wide range of cell types even though variation in abundance is often observed.

An "analogue" includes any substitution, deletion, or additions to the sequence of the HD of the present invention provided that the analogue maintains at least one property associated with the activity of HD as described herein. One such property includes repressing gene expression.

The DNA sequences of the present invention include the DNA sequences of SEQ ID NO: 1, 3, 5 and 7 and fragments thereof, as well as analogues of, or nucleic acid sequences comprising about substantial homology of about 80% similarity with the nucleic acids as defined in SEQ ID NO's: 1, 3, 5 and 7. Analogues (as defined above), include those DNA sequences which hybridize under stringent hybridization conditions (see Maniatis *et al.*, in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982, p. 387-389) to any one of the DNA sequence of SEQ ID NO: 1, 3, 5 or 7 provided that said sequences maintain at least one property of the activity of the HD as defined herein.

An example of one such stringent hybridization conditions may be hybridization in 4XSSC at 65°C, followed by washing in 0.1XSSC at 65°C for an hour. Alternatively an exemplary stringent hybridization condition could be in 50% formamide, 4XSSC at 42°C. Analogues also include those DNA sequences which hybridize to any one of the sequences of SEQ ID NO: 1, 3, 5 or 7 under relaxed hybridization conditions, provided that said sequences maintain at least one regulatory property of the activity of the regulatory element. Examples of such non-hybridization conditions includes hybridization in 4XSSC at 50°C or with 30-40% formamide at 42°C. Another set of hybridization conditions include: 65^{0}C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1 mM EDTA, followed by washing for 15 min in 2 x SSC with 0.1% SDS at room temperature, then twice for 20 min in 0.1 x SSC, 0.1% SDS at 65^{0}C.

The present invention is further directed to one or more chimeric gene constructs comprising a gene of interest operatively linked to a regulatory element. Any exogenous gene can be used as a gene of interest and manipulated according to the present invention to result in the regulated expression of the exogenous gene.

The one or more chimeric gene constructs of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic

acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon.

Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (*Nos* gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene. The 3' untranslated region from the structural gene of the present construct can therefore be used to construct chimeric genes for expression in plants.

One or more of the chimeric gene constructs of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence.

To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable markers include enzymes which provide for resistance to chemicals such as an antibiotic for example, gentamycin, hygromycin, kanamycin, or herbicides such as phosphinothrycin, glyphosate, chlorosulfuron, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as *GUS* (β-glucuronidase), or luminescence, such as luciferase are useful.

Also considered part of this invention are transgenic plants containing the chimeric gene construct of the present invention. However, it is to be understood that the chimeric gene constructs of the present invention may also be combined with gene of interest for expression within a range of plant hosts.

By "gene of interest" it is meant any gene that is to be expressed within a host organism. Such a gene of interest may include, but is not limited to, a gene whose product has an effect on plant growth or yield, for example a plant growth regulator such as an auxin or cytokinin and their analogous, or a gene of interest may comprise a herbicide or a pesticide resistance gene, which are well known within the art. A gene of interest may also include a gene that encodes a pharmaceutically active protein, for example growth factors, growth regulators, antibodies, antigens, their derivatives useful for immunization or vaccination and the like. Such proteins include, but are not limited to, interleukins, insulin, G-CSF, GM-CSF, hPG-CSF, M-CSF or combinations thereof, interferons, for example, interferon-α, interferon-ß, interferon-τ, blood clotting factors, for example, Factor VIII, Factor IX, or tPA or combinations thereof. A gene of interest may also encode an industrial enzyme, protein supplement, nutraceutical, or a value-added product for feed, food, or both feed and food use. Examples of such proteins include, but are not limited to proteases, oxidases, phytases, chitinases, invertases, lipases, cellulases, xylanases, enzymes involved in oil biosynthesis etc.

Methods of regenerating whole plants from plant cells are also known in the art. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques. Transgenic plants can also be generated without using tissue cultures (for example, Clough and Bent, 1998)

The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); Geierson and Corey, *Plant Molecular Biology*, 2d Ed. (1988); and Miki and Iyer, Fundamentals of Gene Transfer in Plants. In *Plant Metabolism*, 2d Ed. DT.

Dennis, DH Turpin, DD Lefebrve, DB Layzell (eds), Addison Wesly, Langmans Ltd. London, pp. 561-579 (1997); Clough and Bent (1998)). The present invention further includes a suitable vector comprising the chimeric gene construct.

The present invention relates to chimeric constructs and a method for regulating the expression of a gene of interest through the use of at least one HD. The chimeric constructs include:

- a first chimeric construct (the expression construct) comprising a first regulatory element, a controlling sequence (CS), a gene of interest, and a terminator. The first regulatory element may permit the constitutive, developmental or temporal expression of the gene of interest within a plant; and

- a second chimeric construct (the effector construct), comprising a second regulatory element, a gene encoding a CS binding domain (CS-BD), and HD, and a terminator sequence. The second regulatory element may permit the constitutive, developmental, temporal or induced expression of the HD within a plant.

The method includes introducing the first and second chimeric constructs as described above, within a plant in order to obtain controlled expression of the gene of interest. The introduction of the two chimeric constructs within a plant may take place using techniques well known within the art such as transformation wherein both chimeric constructs are introduced into the same plant, or through mating plants that each comprise one of the desired constructs in order to obtain a plant that expresses both chimeric constructs.

The CS and CS-BD are characterized in that they exhibit an affinity for each other and are capable of interacting *in vivo*. In this manner, the product of the effector construct, comprising CS-BD and HD, is targeted to the CS of the expression construct. Results described herein demonstrate that the activity of the expression construct is repressed through the targeting of an effector construct product comprising HD. While

not wishing to be bound by theory, this repression may result from the localized deacetylation of histones by HD which results in the repression of transcription of the gene of interest.

By "controlling sequence" (or CS) it is meant, a nucleotide sequence, for example, but not limited to, a regulatory region of a gene, that interacts with a DNA binding protein. However, a CS may include any nucleotide sequence that interacts with a DNA binding protein. The CS is preferably located in proximity with the gene of interest, either upstream or downstream of the gene. An example of a CS and CS-BD may include, but are not limited to, the GAL4 binding domain (GAL4-BD) and the GAL4 upstream activating sequence (GAL4-UAS). However, it is to be understood that other recognition sequences may be used for this purpose as are known to one of skill within the art. For example, a CS may be an endogenous CS associated with a gene, that is involved within a gene expression cascade, for example but not limited to a developmental cascade. In this embodiment the CS is preferably associated with a gene that is involved at an early stage within the gene cascade, for example homeotic genes. Examples of CS and CS-DB's that are involved in initiating a gene cascade, including homeotic genes are well known to one of skill in the art and include, but are not limited to, transcription factor proteins and associated regulatory regions, for example controlling sequences that bind AP2 domain containing transcription factors, for example, APETALA2 (a regulator of meristem identity, floral organ specification, seedcoat development and floral homeotic gene expression; Jofuku *et al.*, 1994), PRbox (pathogen resistance binding proteins), and several stress induced DNA binding proteins, or CCAAT box-binding transcription factors (e.g. *LEC*1; WO 98/37184; Lotan, T., et al.,1998, Cell 93, 1195-1205). Other examples which are not to be considered limiting in any manner of such a regulatory region include BNM3, a regulator of embryogenesis (EP 99201745.9-2105; filed June 2, 1999), or the controlling factor associated with PICKLE, a gene that produces a thickened, primary root meristem (Ogas, J., et al,.1997, Science 277, 91-94.)

The first and second regulatory elements denoted above, may be the same or different. For example, which is not to be considered limiting in any manner, the second regulatory element may be active before, during, or after the activity of the first regulatory

element thereby either initially repressing expression of the gene of interest followed by permitting the expression of the gene of interest, or, following expression of the gene of interest, the second regulatory element becomes active which results in the repression of the expression of the gene of interest. Other examples, which are not to be considered limiting, include the second regulatory element being an inducible regulatory element that is activated by an external stimulus so that repression of gene expression may be controlled through the addition of an inducer. The second regulatory element may also be active during a specific developmental stage preceding, during, or following that of the activity of the first regulatory element. In this way the expression of the gene of interest may be repressed or activated as desired within a plant (see Example 4 and 5).

It is also within the scope of the present invention that the chimeric construct may comprise the elements of the expression construct, as described above, and those of the effector construct, as described above, in a contiguous manner, so that all of the elements for expressing a gene of interest and expressing HD are provided for on one chimeric construct. The first and second regulatory regions may be the same or different, and selected to provide for the constitutive, developmental, temporal or induced expression of either the gene of interest or HD as desired.

The present invention is also directed to a method of regulating gene expression in a transgenic plant that involves the use of only one chimeric construct comprising HD. For example, a method for regulating gene expression may involve:

i) introducing into a plant a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase and a nucleotide sequence encoding a controlling sequence binding protein, to produce said transgenic plant; and

ii) growing the transgenic plant,

wherein the controlling sequence binding protein has an affinity for a native nucleotide sequence within said plant (see Example 6). Preferably the controlling sequence binding protein, for example, but not limited to aDNA binding protein, has an affinity for a

controlling sequence, for example a UAS. By selectively binding the controlling sequence, the chimeric construct results in localized deacetylation of histones by HD which results in the repression of transcription of the gene involved in initiating a gene expression cascade.

This invention also pertains to a method for identifying an endogenous DNA binding protein comprising:

i) introducing into an organism a chimeric nucleotide sequence comprising a nucleotide sequence encoding histone deaceytlase and a marker;
ii) growing the organism;
iii) screening mutants that exhibit a mutant phenotype and assaying for the presence of the marker to obtain a mutant organism; and
iv) isolating a nucleotide sequence comprising the endogenous DNA binding protein from said mutant organism.

With this method a "promoterless" HD randomly inserts within the host DNA. Several of these insertion events result in the HD lying within the vicinity of an endogenous DNA binding protein. The occurrence of the production of a DNA binding protein-HD chimera represses the expression of the gene typically mediated by the DNA binding protein through the interaction of the DNA binding protein and the controlling sequence. Such an event will result in a mutant phenotype that may then be correlated with the occurrence of the HD-marker within the mutant. Once such a mutant phenotype is identified, the adjacent nucleotide sequence may be obtained using the nucleotide sequence encoding the HD, marker, or both the HD and marker, and the DNA binding protein identified. The controlling sequence may also be identified via methods known within the art, for example South-Western analysis.

The HD of the present invention may also be used for altering the development of an organism. This method comprises:

i) transiently introducing into an organism a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase and a nucleotide sequence encoding a DNA binding protein specific for a controlling sequence; and

ii) growing the organism.

With this method, by binding a controlling sequence and repressing the expression of its associated gene via HD, the development of the organism may be altered. Preferably the UAS and associated gene are involved at an early stage within the developmental cascade. As a result only the transient expression of the chimeric nucleotide construct comprising HD is required. Such methods for transient expression are well known in the art, and include, but are not limited to viral transformation, or particle bombardment systems (Klein, T.M., Wolf, E.D., Wu, R. and Sanford, J.C.,1987, Nature 327,70-73, which is incorparated by reference).

To identify *RPD3* and *HD2* homologues in *Arabidopsis thaliana*, a screen of the *Arabidopsis* expressed sequence tags (ESTs) database was performed with either the yeast *RPD3* sequence or the maize *HD2* sequence. Two EST clones were identified corresponding to the yeast *RPD3* sequence and two clones were identified corresponding to the maize *HD2* sequence. These clones were termed *AtRPD3A* (SEQ ID NO:1), *AtRPD3B* (SEQ ID NO:3), *AtHD2A* (SEQ ID NO:5) and *AtHD2B* (SEQ ID NO:7), respectively. The deduced amino acid sequences of *AtRPD3A* (SEQ ID NO: 2) and *AtRPD3B* (SEQ ID NO:4) showed high levels of overall homology to each other (55% identity). Analysis of the sequence of *AtRPD3A* revealed the existence of an ORF (open reading frame) of 1509 base pairs encoding a putative protein of 502 amino acids (SEQ ID NO:2; Figure 1(A)).

To investigate the copy number of *AtRPD3A*, *AtRPD3B*, *AtHD2A* and *AtHD2B* genes in the *Arabidopsis* genome, ^{32}P-labeled *AtRPD3A*, *AtRPD3B*, *AtHD2A* and *AtHD2B* cDNA probes were hybridized to *Arabidopsis* genomic DNA digested with *Eco*RI, *Hin*dIII, *Pst*I and *Xho*I restriction enzymes (Figures 5 and 6). One single band or

two bands were observed in each lane, indicating that *AtRPD3A*, *AtRPD3B*, *AtHD2A* and *AtHD2B* genes are present as a single copy in the *Arabidopsis* genome.

The expression levels of *AtRPD3A and AtRPD3B* transcripts in *Arabidopsis* plants were analyzed by Northern hybridization. As shown in Figure 7, *AtRPD3A* RNA accumulated to relatively high levels in the leaves, stems, flowers and young siliques. *AtRPD3B* RNA, however, was not detectable under the same experimental conditions (data not shown), suggesting that *AtRPD3B* was not expressed or expressed at a very low level in these organs.

The pattern of *AtHD2A and AtHD2B* RNA expression in *Arabidopsis* plants was similarly analyzed by Northern hybridization (Figure 8). *AtHD2A* RNA accumulated in the flowers and young siliques. *AtHD2B* RNA, however, accumulated to relative high levels in the stem, flowers and young siliques and to a somewhat lower level in the leaves.

Repression of Gene Expression by *AtRPD3A* and *AtHD2A*

To determine if *Arabidopsis* RPD3-like and HD-like proteins possess gene repression activity, effector plasmids were constructed. A GAL4-*AtRPD3A* effector plasmid was designed and constructed in which the *AtRPD3A* protein was fused with the DNA-binding domain of the yeast transcription factor GAL4 (Giniger et al., 1985; Ma et al., 1988) and driven by full length tCUP, a strong constitutive promoter (Foster et al., 1999) (See Figure 9(A) for effector and reporter constructs). A reporter plasmid, UAS_{GAL4}-tCUP-GUS, was constructed using a GUS reporter gene and in which two GAL4-binding sites (UAS_{GAL4}) were fused to a truncated tCUP promoter, -394-tCUP (Foster et al., 1999). Each of the effector plasmids, either GAL4-*AtRPD3A* or GAL4, was co-bombarded into tobacco leaves together with the reporter plasmid UAS_{GAL4}-tCUP-GUS, the idea being that the fusion protein would target *AtRPD3A* to promoters containing the GAL4-binding sites (UAS_{GAL4}). In the case of the control, the reporter plasmid was co-bombarded with the control plasmid pUC19..

As shown in Figure 9 (B) the levels of GUS activity were essentially the same when the reporter plasmid was co-bombarded with either the control plasmid pUS19, or GAL4 effector plasmid. However, an approximate 2-fold repression in GUS activity was observed in the presence of GAL4-*AtRPD3A* when compared with the other two treatments. This demonstrates that the protein product of *AtRPD3A* gene expression is capable of mediating transcriptional repression of transgenic sequences and suggest that transcriptional repression occurs by targeted histone deacetylation and the establishment of a locally repressive chromatin structure.

Similar constructs were tested in a like manner for *AtHD2A* proteins. A reporter plasmid was constructed with a GUS reporter gene (UAS_{GAL4}-tCUP-GUS) in which GAL4-binding sites (UAS_{GAL4}) were fused to the strong constitutive promoter -394-tCUP (Foster et al., 1999). The reporter gene was designed to be repressed by the fusion protein encoded by the effector plasmid GAL4-*AtHD2A*. As shown in Figure 10 (A), the *AtHD2A* protein (*AtHD2A*, 1-245) was fused with the DNA-binding domain of the yeast transcription factor GAL4 (GAL4BD) (Giniger et al., 1985; Ma et al., 1988). Several deletions of *AtHD2A* were also prepared and tested in association with the reporter construct. Each of the effector plasmids also contained the 35S promoter. Tobacco leaves were co-bombarded with the reporter construct and either GAL4, one of the AtHD2A effector plasmids, or the control plasmid pUS19, and GUS activity determined.

Co-bombardment of leaves with either reporter and the control construct or reporter and GAL4 resulted in a high level of GUS activity (Figure 10 (B)), while co-bombardment with AtHD2A significantly reduced GUS activity. These results again indicate that *AtHD2A* can mediate transcriptional repression of a targeted reporter gene *in vivo*. To determine the protein domains of *AtHD2A* responsible for gene repression, a series of deletion constructs of *AtHD2A* were made (Figure 10 (B) and tested by transient expression in Arabidopsis plants. Deletion of C-terminal residues up until the amino acid 162 of *AtHD2A* (GAL4-*AtHD2A*, 1-211 and GAL4-*AtHD2A*,1-162) did not affect the repression activity of the molecules (Figure 10 (B). However, further deletions to the amino acid 100 of the C-terminal residue (GAL4-*AtHD2A*, 1-100) resulted in a complete

loss of gene repression activity. This observation indicate that the region between the amino acid residues 101 to 161 is important for repression activity. This region also includes an extensive acidic amino acid domain, which is important for association with basic tails of histones (Philpott and Leno, 1992). Deletion of the domain containing predicted catalytic residues (GAL4-*AtHD2A*, 73-245) resulted in complete loss of repression activity (Figure 10 (B)). Collectively, these results demonstrate that both the deacetylase catalytic activity and HD binding with histones is essential for gene repression activity. Furthermore, these results indicate that fragments or analogs of HD are active in repressing the expression of a gene of interest.

Antisense Expression of AtRPD3A and AtHD2A

The difference in *AtRPD3A* and *AtRPD3B* expression suggests that these genes and their corresponding proteins might function in different roles during plant growth and development. To test this hypothesis, *Arabidopsis* plants were transformed with an antisense construct of the *AtRPD3A* cDNA. Transgenic *Arabidopsis* plants were generated that expressed antisense *AtRPD3A* driven by the strong constitutive promoter, -394-tCUP (Foster et al., 1999). A truncated 519 bp fragment of *AtRPD3A* cDNA driven by -394-tCUP promoter was used to make an antisense construct (Figure 11). The expression of antisense *AtRPD3A* RNA in the transgenic lines was monitored by Northern analysis (Figure 12). Because a truncated *AtRPD3A* cDNA was used to make the antisense constructs, it was expected that the antisense transcript would be smaller than the endogenous sense transcript. Indeed, two transcripts, a large transcript (1.6 kb) and a smaller transcript (0.6 kb), were detected using an *AtRPD3A* cDNA probe in the antisense transgenic lines (Figure 12). The smaller transcript was absent from the wild-type plants and represented the *AtRPD3A* antisense transcript. As shown in Figure 12, different levels of endogenous sense *AtRPD3A* transcript were detected in three independent antisense lines with high levels of expression of antisense transcript. Two antisense lines, B5 and A1, also showed a considerable reduction of endogenous transcripts compared with wild-type.

The two independent antisense lines with reduced endogenous *AtRPD3A* transcripts had a delayed- flowering phenotype compared with wild-type plants. As shown in Figure 14, control wild-type plants started flowering after approximately 3 weeks of vegetative growth. In contrast, flowering of the antisense lines A1 and B5 was delayed for two to three weeks. These results suggest that *AtRPD3A* is important for normal plant growth and development.

In *Arabidopsis*, the difference in *AtHD2A* and *AtHD2B* expression patterns also suggests that these genes and their corresponding proteins may play different roles during normal plant growth and development. *AtHD2B* appears to be expressed constitutively in *Arabidopsis*. Whereas *AtHD2A*, shows a restricted pattern of expression within the flowers and siliques.

To further study the function of HD2-like proteins, transgenic *Arabidopsis* (ecotype Columbia) were constructed. These plants that expressed antisense *AtHD2A* from a strong constitutive promoter, -394-tCUP. The expression of the *AtHD2A* antisense gene in the transgenic lines was verified by Northern analysis. An antisense specific probe derived from the 5' untranslated region of the *AtHD2A* antisense construct was used to monitor the expression of the *AtHD2A* antisense gene. As shown in Figure 13, five independent transgenic lines showed high expression of antisense *AtHD2A* transcript. A 3' untranslated region of *AtHD2A* cDNA, which was absent from the *AtHD2A* antisense construct was used to detect the endogenous *AtHD2A* mRNA. As shown in Figure 13, the levels of endogenous *AtHD2A* transcript were significantly reduced in the transgenic lines, suggesting that antisense transcripts might trigger *AtHD2A* mRNA degradation.

The five independent transgenic lines with reduced endogenous *AtHD2A* RNA levels had stunted siliques and produced fewer seeds compared with wild-type (Fig. 15), and they were therefore semi-sterile. The wild-type plants were distinguished from the transgenic plants by the length of the siliques and the seed set. In the semi-sterile transgenic plants, silique length and seed set varied along the stem and from

inflorescence to inflorescence. Siliques from the wild type and the sterile transgenic plants were dissected and examined by stereomicroscopy and scanning electron microscopy. As shown in Figure 16, the transgenic mature siliques contained aborted seeds, which were significantly smaller than the healthy seeds from the wild-type plants.

Antisense expression of histone deacetylase genes may have resulted in an alteration in the chromatin structure by hyperacetylation of histones, which subsequently affected gene transcription. Our study indicates that histone deacetylases play an important role in regulating different developmental pathways of plants and that the developmental abnomalities seen in deacetylase antisense plants may be due to dysregulation of gene expression.

Tissue specific gene repression

Tissue-specific repression of gene expression is also observed in plants using tissue-specific regulatory elements to drive the expression of either the expression, effector, or bothe the expression and effector construct. As described herein, which is not to be considered limiting in any manner, seed specific expression of either the expression or effector construct may be obtained using the napin promoter. Results demonstrate that repression of an expression construct under the control of a constitutive regulatory element in a tissue-specific manner may be obtained by either crossing plants expressing the expression construct with plants expressing tissue-specific expression of the effector construct (see Example 4), by sequentially transforming plants with an expression construct, and then re-transforming the plant with an effector construct to produce a dual transgenic plant (see Example 5), or co-transforming a plant with both the expression and effector construct at the same time (e.g. Example 2). An outline of the experimental approach for crossing plants expressing an expression construct with plants expressing an effector construct to produce a dual transgenic plant is presented in Figure 17 (A). Non-limiting examples of constructs that exemplify this proceedure are schematically presented in Figure 17

(B), however, it is to be understood that other expression-effector constructs may be used to drive repression of a desired gene in a plant.

Plants transformed with UAS_{GAL4}-tCUP-GUS were crossed with either tCUP-GAL4/AtHD2A (constitutive expression) and NAP-GAL4/AtHD2A (tissue specific expression) effector lines. Analysis of the F1 progeny from a cross between UAS_{Gal4}-tCUP-GUS X 35S-GAL4/AtHD2A, and UAS_{Gal4}-tCUP-GUS X NAP1-GAL4/AtHD2A are presented in Figure 19 (A). High levels of expression of a gene of interest (e.g. reporter gene activity) are observed in leaves and seeds in control plants expressing GUS under the control of the constitutive regulatory element tCUP. In F1 progeny of plants derived from a cross between UAS_{Gal4}-tCUP-GUS X 35S-GAL4/AtHD2A, reduced reporter gene expression is observed in both leaves and seeds, due to the constitutive expression of the HD/GAL4BD, and the UAS_{Gal4}-reporter genes. High levels of expression of a gene of interest (e.g. a reporter gene) are observed in leaf tissue of F1 progeny derived from a cross between UAS_{Gal4}-tCUP-GUS X NAP1-GAL4/AtHD2A due to a lack of expression of the effector construct under the control of the tissue-specific promoter. However, in seed tissues, reporter expression is dramatically reduced due to the targeted expression of the HD/GAL4BD.

Similar results are obtained in dual transgenic plants that have been transformed sequentially, that is, following the initial transformation of a plant with an expression gene (for example GUS), the transgenic plant is re-transformation with an effector gene. As shown in Figures 19 (B) and (C) plants transformed with both an expression construct and re-transformed with either 35S-GAL4/AtHD2A, or NAP1-GAL4/AtHD2A display similar patterns of repression of the gene of interest as that observed following crossing expression X effector plant lines. In plants sequentially transformed with the reporter construct and an effector construct that is constitutively expressed in the plant (35S-GAL4/HD), repression of GUS activity is observed in both leaves and seed (Figures 19 (B) and (C)). Repression of GUS activity is only observed in seed tissues in dual transgenic plants sequentially transformed with the expression construct followed by the seed specific effector construct NAP1-GAL4/HD. No

repression of reporter gene activity was observed in leaf tissue in dual transgenic plants re-transformed with the seed specific effector construct.

Plant Transcription Factors may be used to Repress Developmental, Physiological, or Biochemical Pathways

The methods as described herein may also be used to repress developmental, physiological and metabolic pathways in plants. In this embodiment, protein factors bind specific DNA sequences within a regulatory region of a gene. These protein factors function as a controlling sequence binding domain (CS-BD), and the specific DNA sequence to which the CS-BD binds function as a controlling sequence (CS).

An example, which is not to be considered limiting in any manner, involves the use of an effector construct comprising HD associated with a CS-BD. The CS-BD, for example, but not limited to a transcription factor, is capable of binding an endogenous CS within the plant, thereby permitting the associated HD to repress expression of a gene associated with the CS. If the CS is associated with a gene involved with a developmental or metabolic cascade, for example but not limited to a homeotic gene, then repression of the gene of interest ensures that the cascade is not initiated.

An example to demonstrate that developmental, physiological or biochemical pathways can be regulated by the methods as disclosed herein, involves the repression of a developmental or metabolic pathway associated with the ethylene response in plants using the transcriptional factor Pti4 fused with histone deacetylase.

Pti4 is a tomato transcription factor that belongs to the ERF (ethylene-responsive element binding factor) family of proteins. It interacts with the Pto kinase in tomato, which confers resistance to the *Pseudomonas syringae* pv *tomato* pathogen that causes bacterial speck disease. To study the function of Pti4, transgenic *Arabidopsis* plants were generated that expressed tomato *Pti4* driven by the strong constitutive promoters, CaMV 35S and -394tCUP.

Pti4 enhanced GCC box-mediated transcription of a gene of interest with which it was co-transformed (Figure 20). Solano et al. (1998) reported that overexpression of another ERF (ethylene-responsive element binding factor) protein, ERF1, in transgenic *Arabidopsis* plants induced basic chitinase gene expression. Basic chitinase is an ethylene-responsive gene, which contains the GCC box in its promoter (Samac et al., 1990). The GCC-box contains a conserved AGCCGCC sequence, which was first identified from the promoters of ethylene-inducible PR genes in tobacco (Ohme-Takagi and Shinshi, 1995). Without wishing to be bound by theory, it has been suggested that this sequence is a target in the ethylene signal transduction pathway because deletion of the GCC box eliminates ethylene responsiveness (Broglie et al., 1989;; Shinshi et al., 1995). Therefore, the expression of tomato *Pti4* in *Arabidopsis* was examined to determine if Pti4 could induce the expression of the *Arabidopsis* basic chitinase gene. Northern analysis also showed that expression of *Pti4* in transgenic *Arabidopsis* plants induced the expression of a GCC box-containing, endogenous, PR gene, basic chitinase, in *Arabidopsis* (Figure 21).

The ethylene-responsive phenotype is exhibited in Arabidopsis by an inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl and root, and exaggeration in the curvature of the apical hook (Ecker, 1995; Chang and Shockey, 1999). The hypocotyls of etiolated transgenic seedlings were measured 72 hrs after germination. Plants expressing *Pti4*/HD construct showed inhibition of hypocotyl elongation, a phenotype similar to those observed in the constitutive ethylene response-mutants or in wild-type plants exposed to ethylene (Solano et al., 1998). As shown in the Figures 22 and 23, the seedlings from the transgenic line tCUP/Pti4-1 displayed strong inhibition of hypocotyl elongation similar to that seen in plants treated with ethylene (ACC), suggesting that the *Pti4* gene is involved in the regulation of a subset of ethylene responsive genes which contain the GCC box, and indicate that tomato Pti4 acts as a transcriptional activator to regulate expression of GCC box-containing genes.

To test the effect of Pti4-AtHD2A protein on the ethylene signaling pathway, transgenic plants overexpressing *Pti4-AtFD2A* were examined for the ethylene-responsive phenotype. The hypocotyls of the etiolated transgenic seedlings were

measured 72 hrs after germination. As shown in the Figures 24, wild type seedlings (Figure 24 (A)) exhibited hypocotyl elongation. Seedlings overexpressing Pti4 (Figure 24 (B) exhibited the ethylene responsive phenotype (inhibition of hypocotyl elongation). However, seedlings from the transgenic line Pti4-HDA (Figure 24 (C)) did not display inhibition of hypocotyl elongation, demonstrating that Pti4-AtHDA fusion proteins repressed ethylene responsive phenotype in transgenic plants.

These results indicate that the Pti4 functions as a CS-BD (controlling sequence-binding domain) and is capable of interacting with a controlling sequence (GCC box), and target HD to repress gene expression of an endogenous gene in a gene-specific manner.

Therefore the present invention is directed to a method of regulating the expression of a gene of interest in a plant comprising:

i) introducing into the plant a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase fused with a nucleotide sequence encoding a controlling sequence binding domain that has an affinity for a native controlling sequence upstream within the gene, to produce a transgenic plant; and

ii) growing the transgenic plant.

The controlling sequence binding domain may be for example A DNA binding protein, and the controlling sequence may be an upstream activating sequence.

Similarly, the above method may be used to regulate a developmental, physiological, or biochemical pathway within a plant by introducing into the plant a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase fused with a nucleotide sequence encoding a DNA binding protein that has an affinity for a native upstream activating sequence within the gene of interest known to be associated with a developmental, physiological or biochemical cascade, for example a homeotic gene, to produce a transgenic plant.

Identification of DNA binding proteins

The method of repressing gene expression as disclosed herein may also be used as a functional test for identifying a phenotype associated with perturbing a gene comprising a controlling sequence, as well as identifying controlling sequences, upstream activating sequences, controlling sequence binding domains, transcription factors, or DNA binding proteins in general.

In this method, a nucleotide sequence of unknown function, for example a putative transcription factor, can be tested to see if it targets the repression of gene expression, when fused with a histone deacetylase. If an altered phenotype can be determined as a result of the introduction of the construct into the plant, then this indicates that the unknown nucleotide sequence interacts with a controlling sequence associated with a gene in such as manner so as to permit HD to modify/repress the expression of the gene. Such a functional test can be used to screen nucleotide sequences thought to comprise DNA binding proteins, and determine the associated phenotype arising from repressing expression of the gene comprising the controlling sequence..

Therefore, the present invention also provides for a method for identifying DNA binding protein comprising:

i) introducing into the plant a chimeric nucleotide sequence comprising a regulatory element in operative association with a nucleotide sequence encoding histone deaceytlase fused with a nucleotide sequence of interest and of unknown function (e.g. a putative DNA binding protein), to produce a transgenic plant;

ii) growing the transgenic plant; and

iii) examining the phenotype of the transgenic plant to determine whether the chimeric nucleotide sequence, comprising the nucleotide sequence of interest has an effect on the plant phenotype.

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

Example 1:

Growth of Plant Material

Arabidopsis thaliana (ecotype Columbia) was grown in a growth chamber (16 hr of light and 8 hr of darkness at 23°C) after a 2-4 day vernalization period. For growth under sterile conditions, seeds were surface sterilized (15 min incubation in 5% [v/v] sodium hypochlorite, and a threefold rinse in sterile distilled water) and sown on half-strength Murashige and Skoog (MS) salts (Sigma) (Murashige and Skoog, 1962) supplemented with 1% sucrose, pH 5.7, and 0.8% (w/v) agar in Petri dishes.

Isolation of *AtRPD3* and AtHD2

To identify *RPD3* and *HD2* homologues in *Arabidopsis thaliana*, a screen of the *Arabidopsis* expressed sequence tags (ESTs) database was performed with either the yeast *RPD3* sequence or the maize *HD2* sequence. Two EST clones were identified corresponding to the yeast *RPD3* sequence and two clones were identified corresponding to the maize *HD2* sequence. The clones were termed *AtRPD3A*, *AtRPD3B*, *AtHD2A* and *AtHD2B*, respectively.

DNA and Protein Sequence Analysis

Dye primer sequencing of cDNA clone inserts and dye terminator sequencing of PCR products were performed using an automated sequencing system (Applied Biosystems). DNA and protein sequence analysis was carried out using BLAST searches (Altschul et al., 1990) and the DNASIS program (Hitachi Software Engineering Co., Ltd).

The isolated clones were sequenced *AtRPD3A* (SEQ ID NO:1), *AtRPD3B* (SEQ ID NO:3), *AtHD2A* (SEQ ID NO:5) and *AtHD2B* (SEQ ID NO:7), respectively (see also Figures 1 and 2).

The sequence of cDNA clone *AtRPD3B* is truncated at the 5' extremity in comparison to *AtRPD3A*. The genomic sequence of *AtRPD3B* was identified on chromosome 5 of *Arabidopsis* (GenBank accession no. AB008265). The sequence of *AtRPD3B* encodes an ORF of 1416 base pairs encoding a putative protein of 471 amino acids (SEQ ID NO:4; Figure 1(B)).

Analysis of the sequences of the *AtHD2A* revealed an ORF of 738 base pairs encoding a putative protein of 245 amino acids whereas the sequence of the *AtHD2B* contained an ORF of 918 base pairs encoding a putative protein of 305 amino acids (SEQ ID NO's: 6 and 8, respectively; Figure 2).

The deduced protein sequences of *AtRPD3A* and *AtRPD3B* were aligned with yeast *RPD3*, and the maize *RPD3* homolog, ZmRPD3 (Rossi et al., 1998). As shown in Figure 3, *AtRPD3A* is more closely related to maize *ZmRPD3* (73% identity) than to yeast *RPD3* (49% identity). *AtRPD3B*, however, shows 57% and 55% amino acid identity with maize *ZmRPD3* and yeast *RPD3*, respectively. The putative residues essential for histone deacetylase activity (Hassig et al., 1998) were strictly conserved in all of these proteins (Figure 3).

The deduced protein sequences of *AtHD2A* and *AtHD2B* was aligned with maize *HD2*, *ZmHD2* (Lusser et al., 1997; Figure 4). The *AtHD2A* and *AtHD2B*

sequences share 52% amino acid identity to each other, and they share 44% and 46% amino acid identity with the *ZmHD2*, respectively. As shown in Figure 4, the predicted histone deacetylase catalytic residues (Aravind and Koonin, 1998) are conserved in the N-terminal domains of both *AtHD2A* and *AtHD2B*. Similarly, both proteins contain an extended acidic amino acid domain, with high sequence homology to nucleolar proteins from several organisms (Lusser et al., 1998). Additionally, a putative zinc finger is encoded at the C-terminal domain of *AtHD2A*, but not at the C-terminal of *AtHD2B*.

Southern and Northern Blot Analysis

Total genomic DNA from *Arabidopsis* was extracted as described (Dellaporta et al. 1983). For Southern blots, *Arabidopsis* genomic DNA was digested with restriction enzymes, separated by agarose gel electrophoresis, and transferred to nylon membranes (Sambrook et al., 1989). For Northern analysis, total RNA was isolated from 100-200 mg *Arabidopsis* tissues using Tri™Pure Reagent as described by the manufacturer (Boehringer Mannheim). Northern blots were prepared by electrophoresis of 5-10 μg samples of total RNA through agarose gels in the presence of formaldehyde (Strommer et al., 1993), followed by transfer to nylon membranes. Southern and Northern blots were probed with ^{32}P-labeled probes. Prehybridization and hybridization were performed at 65°C in 0.5 M Na_2HPO_4 (pH 7.2), 7% SDS, and 1 mM EDTA. Filters were washed once for 15 min in 2 x SSC with 0.1% SDS at room temperature, then twice for 20 min in 0.1 x SSC, 0.1% SDS at 65°C. The damp filters were autoradiographed at -80°C using two intensifying screens. Filters were stripped in 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS at 100°C for 2 min when reprobing was required. As a control, all Northern blots were also probed with an *Arabidopsis* actin (EST clone 40F11 from the *Arabidopsis* Biological Resource Center, Ohio State University).

To investigate the copy number of *AtRPD3A*, *AtRPD3B*, *AtHD2A* and *AtHD2B* genes in the *Arabidopsis* genome, ^{32}P-labeled *AtRPD3A*, *AtRPD3B*, *AtHD2A* and

AtHD2B cDNA probes were hybridized to *Arabidopsis* genomic DNA digested with *Eco*RI, *Hin*dIII, *Pst*I and *Xho*I restriction enzymes (Figures 5 and 6). One single band or two bands were observed in each lane, indicating that *AtRPD3A*, *AtRPD3B*, *AtHD2A* and *AtHD2B* genes are present as a single copy in the *Arabidopsis* genome.

The expression levels of *AtRPD3A*, *AtRPD3B*, *AtHD2A* and *AtHD2B* transcripts in *Arabidopsis* plants were analyzed by Northern hybridization (Figures 7 and 8). *AtRPD3A* RNA accumulates to relatively high levels in the leaves, stems, flowers and young siliques. *AtRPD3B* RNA, however, was not detectable under the same experimental conditions (data not shown), suggesting that *AtRPD3B* was not expressed or expressed at a very low level in these organs (Figure 7). *AtHD2A* RNA accumulated in the flowers and young siliques. *AtHD2B* RNA, however, accumulated to relative high levels in the stem, flowers and young siliques and to a somewhat lower level in the leaves (Figure 8).

Example 2:

Construction of Transformation Plasmids

To construct a reporter construct (an expression construct), the CaMV 35S promoter of pBI221 (Clontech) was replaced with a truncated tCUP promoter, -394-tCUP (Foster et al., 1999) to generate the pBI-BtCUPvector. A 76-bp fragment (CGGAGGACTGTCCTCCGATCGGAGGACTGTCCTCCGTGCA: SEQ ID NO: 9) containing two upstream activating sequence of the yeast GAL4 protein (UAS_{GAL4}) was ligated into the PstI site located upstream of the -394-tCUP promoter.

***AtRPD3A* Effector Plasmids**

To construct the effector plasmids, we replaced the 35S promoter of pBI221 with the tCUP promoter (Foster et al., 1999), to generate the pBI-tCUP vector. The GUS gene in the pBI-tCUP was replaced with the *AtRPD3A* coding region, and the

DNA-binding domain of GAL4 (amino acids 1-147) was subcloned in-frame into the XbaI and XmaI sites.

AtHD2A Effector Plasmids

To construct the effector plasmids, the GUS gene in the pBst-tCUP was replaced with the *AtHD2A* and its deletions, then the DNA binding domain of GAL4 was subcloned in-frame into the XbaI and XmaI sites.

Particle Gun Delivery Assays

Tobacco (SR1) plants were maintained *in vitro* in half-strength MS medium (Murashige and Skoog, 1962) in Magenta containers (Magenta Corp., Chicago) in a growth chamber at 25^{0}C. After transfer to fresh medium for two to three weeks, uniform-sized leaves (about 3 cm in width) were cut off from the plants and placed on a medium consisted of MS salts, B5 vitamins (Gamborg et al., 1968), 1 mg/L 6-benzyladenine, 0.1 mg/L naphthalene acetic acid, 3% sucrose and 0.25 % Gelrite in a 20 x 15 mm Petri dish. The leaves were preconditioned on this medium for one day prior to gene delivery.

Plasmid DNA was isolated using a QIAGEN Plasmid Midi Kit. The reporter plasmid was mixed with an effector plasmid at 1:1 ratio (weight). In the control, the reporter was mixed with an equal amount of control plasmid pUS19. A modified particle inflow gun (Brown et al., 1994) was used for DNA delivery. DNA was precipitated onto tungsten particles by using the following protocol: a 5 μl sample of mixed DNA (1 $\mu g/\mu$) was added to 25 μl tungsten particles (100mg/ml) and followed by the addition of 25 μl of 2.5 M $CaCl_2$ and 5 μl of 0.1 M spermidine. The leaves were bombarded once at a distance 16 cm from the screen and under the pressure of 1000 kPa He gas.

Bombarded leaves were maintained on the same medium for 1 day. Gene expression was determined by histochemical and fluorometric assays (Jefferson 1988).

GUS activity was reported as picomoles of 4-methylumbelliferone per milligram of protein per minute.

Repression of Gene Expression by *AtRPD3A* and *AtHD2A*

Each of the effector plasmids (as described above), either GAL4-*AtRPD3A* or GAL4, was co-bombarded into tobacco leaves together with the reporter plasmid UAS_{GAL4}-tCUP-GUS (as described above). In the case of the control, the reporter plasmid was co-bombarded with the control plamsid pUS19.

As shown in Figure 9 (B) an approximate 2-fold repression in GUS activity was observed when GAL4-*AtRPD3A* was co-bombarded with the reported construct, when compared with the the control or GAL4 contructs. These results indicate that the protein product encoded by *AtRPD3A* is capable of mediating transcriptional repression of transgenic sequences.

Similar constructs, prepared as indicated above, were tested in a like manner for *AtHD2A* proteins. Co-bombardment of leaves with either reporter and control construct or reporter and GAL4 resulted in a high level of GUS activity (Figure 10 (B)). Co-bombardment with AtHD2A resulted in reduced GUS activity. These results again indicate that *AtHD2A* mediates transcriptional repression of a targeted reporter gene *in vivo*.

A series of deletion constructs of *AtHD2A* were made (Figure 10 (B) and tested by transient expression in Arabidopsis plants. Deletion of C-terminal residues up until the amino acid 162 of *AtHD2A* (GAL4-*AtHD2A*, 1-211 and GAL4-*AtHD2A*,1-162) did not affect the repression activity of the molecules (Figure 10 (B). Deletion of the domain containing predicted catalytic residues (GAL4-*AtHD2A*, 73-245) resulted in complete loss of repression activity (Figure 10 (B)). Furthermore, GAL4-*AtHD2A*, 1-100 (with deletions to the amino acid 100 of the C-terminal residue) resulted in a

complete loss of gene repression activity. This region includes an extensive acidic amino acid domain, which is known to intereact with basic tails of histones.

Collectively, these results demonstrate that both the deacetylase catalytic activity and HD binding with histones is essential for gene repression activity. Furthermore, these results indicate that fragments or analogs of HD are active in repressing the expression of a gene of interest.

Example 3: Antisense Constructs

To generate the antisense constructs, pBI-BtCUP was digested with *EcoR*I and HindIII, and the resulting fragment containing the -394-tCUP promoter and the GUS gene was then subcloned into the multi-cloning sites of pCAMBIA2300 binary vector (Cambia, Canberra, Australia) to generate the pCBtCUP vector. *AtRPD3A* cDNA was digested by *Sac*I and *Ssp*I, and the resulting 519 bp fragment of the truncated *AtRPD3A* cDNA in the antisense orientation was used to replace the GUS gene of the pCBtCUP.

To generate *AtHD2A* antisense construct, the *AtHD2A* cDNA fragment was obtained using the polymerase chain reaction procedure. The 738 bp of full-length *AtHD2A* cDNA was amplified in two primer pairs (AATTGAGCTCAGCCATGGAGTTCTGGGG: SEQ ID NO:10 and ACGTGGATCCAGAAACCACTTCACTTGGC: SEQ ID NO:11). All primers had additional nucleotides at the 5' ends to give suitable restriction sites for cloning of the resulting fragments. The PCR product was digested by *Sac*I and *Xma*I and used to replace the GUS gene of pCBtCUP.

Plant Transformation and Selection

Plant transformation plasmids were electroporated into *Agrobacterium* tumefaciens GV3101 (Van Larebeke et al. 1974) as described by Shaw (1995). The Agrobacterium-mediated transformation of *Arabidopsis thaliana* was performed as

described previously (Clough and Bent, 1998), with the following modifications. Plants with immature floral buds and few siliques were dipped into a solution containing *Agrobacterium tumefaciens*, 2.3 g/L MS salts (Sigma), 5% (w/v) sucrose and 0.03% Silwet L-77 (Lehle Seeds, Round Rock, TX) for 1-2 min. T1 seeds were collected, dried at 25°C, and sown on sterile media containing 40 μg/mL kanamycin to select the transformants. Surviving T1 plantlets were transferred to soil to set seeds (T2).

Microscopic Analysis

For scanning electron microscopy, green siliques were dissected under the stereomicroscope and fixed in 4% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH7.2) for 16 hr. The samples were then dehydrated in a graded ethanol series (50-100%). The treated siliques were critical-point-dried using liquid CO_2 and mounted on scanning electron microscope *stubs*. The mounted specimens were coated with gold and observed with a scanning electron microscope.

The expression of antisense *AtRPD3A* RNA in the transgenic lines was monitored by Northern analysis (Figure 12). Two transcripts, a large transcript (1.6 kb) and a smaller transcript (0.6 kb), were detected using an *AtRPD3A* cDNA probe in the antisense transgenic lines (Figure 12). The smaller transcript was absent from the wild-type plants and represented the *AtRPD3A* antisense transcript. As shown in Figure 12, different levels of endogenous sense *AtRPD3A* transcript were detected in three independent antisense lines with high levels of expression of antisense transcript. As shown in Figure 14, control wild-type plants started flowering after approximately 3 weeks of vegetative growth. In contrast, flowering of the antisense lines A1 and B5 was delayed for two to three weeks. These results suggest that *AtRPD3A* is important for normal plant growth and development.

To further study the function of HD2-like proteins, transgenic *Arabidopsis* (ecotype Columbia) were constructed. These plants expressed antisense *AtHD2A* from a strong constitutive promoter, -394-tCUP. The expression of the *AtHD2A* antisense

gene in the transgenic lines was verified by Northern analysis. An antisense specific probe derived from the 5' untranslated region of the *AtHD2A* antisense construct was used to monitor the expression of the *AtHD2A* antisense gene. As shown in Figure 13, five independent transgenic lines showed high expression of antisense *AtHD2A* transcript. A 3' untranslated region of *AtHD2A* cDNA, which was absent from the *AtHD2A* antisense construct was used to detect the endogenous *AtHD2A* mRNA. As shown in Figure 13, the levels of endogenous *AtHD2A* transcript were significantly reduced in the transgenic lines, indicating that antisense transcripts might trigger *AtHD2A* mRNA degradation.

The five independent transgenic lines with reduced endogenous *AtHD2A* RNA levels had stunted siliques and produced fewer seeds compared with wild-type (Fig. 15), and they were therefore semi-sterile. The wild-type plants were distinguished from the transgenic plants by the length of the siliques and the seed set. In the semi-sterile transgenic plants, silique length and seed set varied along the stem and from inflorescence to inflorescence. Siliques from the wild type and the sterile transgenic plants were dissected and examined by stereomicroscopy and scanning electron microscopy. As shown in Figure 16, the transgenic mature siliques contained aborted seeds, which were significantly smaller than the healthy seeds from the wild-type plants.

Example 4: Tissue specific gene repression

The above examples demonstrate repression of GUS reporter gene activity regulated by a constitutive regulatory element fused to a controlling sequence (for example a yeast upstream activating sequence) specific for a controlling sequence binding domain (for example the yeast GAL4 protein DNA binding domain; UAS_{GAL}). This example demonstrates the repression of gene expression in a tissue dependant manner using a tissue-specific regulatory element, for example the napin promoter (a seed specific regulatory element) that drives the expression of the GAL4-HD protein. Results presented below demonstrate that the expression of a target gene that is

regulated by any promoter (including a constitutive promoter) may be repressed in a tissue-specific manner. An outline of the experimental approach is presented in Figure 17 (A). Constructs used are schematically presented in Figure 17 (B).

UAS_{GAL4}-tCUP-GUS and UAS_{GAL4}-35S-GUS reporter constructs (Figure 17 (B)) were used to transform *Arabidopsis* using standard techniques (Clough and Bent,1998) to generate reporter lines. Thirteen UAS_{GAL4}-tCUP-GUS lines and eight UAS_{GAL4}-35S-GUS lines were generated and the expression of GUS gene was screened for GUS activity by histochemical assay. All of thirteen UAS_{GAL4}-tCUP-GUS reporter lines and six of eight UAS_{GAL4}-35S-GUS reporter lines showed intense GUS staining.

Effector lines were generated by using effector plasmids, tCUP-GAL4/AtHD2A and NAP-GAL4/AtHD2A (Figure 15 (B)). Thirteen tCUP-GAL4/AtHD2A and six NAP-GAL4/AtHD2A effector lines were generated. Southern analysis indicated that all of the effector lines carry GAL4/AtHD2A gene (data not shown). Northern analysis indicated that four of the thirteen tCUP-GAL4/AtHD2A effectors showed strong expression of GAL4/AtHD2A mRNA (data not shown).

Three UAS_{GAL4}-tCUP-GUS lines were crossed with three tCUP-GAL4/AtHD2A and NAP-GAL4/AtHD2A effector lines, respectively. Analysis of the F1 progeny from a cross between UAS_{Gal4}-tCUP-GUS X 35S-GAL4/AtHD2A (Effector 1), and UAS_{Gal4}-tCUP-GUS X NAP1-GAL4/AtHD2A (Effector 2) is presented in Figure 19. High levels of reporter gene activity are observed in leaves and seeds in control plants expressing GUS under the control of the constitutive regulatory element tCUP. In F1 progeny of plants derived from a cross between UAS_{Gal4}-tCUP-GUS X 35S-GAL4/AtHD2A (Effector 1), reduced reporter gene expression is observed in both leaves and seeds, due to the constitutive expression of the HD/GAL4BD, and the UAS_{Gal4}-reporter genes. In F1 progeny derived from a cross between UAS_{Gal4}-tCUP-GUS X NAP1-GAL4/AtHD2A (Effector 2), high levels of reporter gene expression are observed in leaf tissue only, with seed specific reporter

expression is dramatically reduced due to the targeted expression of the HD/GAL4BD gene in seed tissues only..

These results indicate that tissue specific repression of gene activity can be achieved though tissue specific expression of a gene encoding a controlling sequence-binding domain.

Example 5: Sequential transformation of plants with target and effector constructs

Repression of a gene of interest may occur within a plant following sequential transformation of a target gene, for example GUS, followed by transformation with an effector gene. To demonstrate the efficacy of this approach, Arabidopsis plants were transformed using standard techniques (Clough and Bent,1998) using the construct UAS_{GAL4}-tCUP-GUS (tCUP-GUS; reporter gene). As shown in Figures 19 (B) and (C), these plants (indicated as control 1, 2) exhibit GUS activity in both leaves and seeds. Transformed plants expressing GUS were then re-transformed with one of two effector constructs, 35S-GAL4/AtHD2A, or NAP1-GAL4/AtHD2A. The levels of GUS activity within the dual transgenics are shown in Figures 19 (B) and (C).

In plants sequentially transformed with the reporter construct and an effector construct that is constitutively expressed in the plant (35S-GAL4/HD), repression of GUS activity was observed in both leaves and seed (Figures 19 (A) and (B)). The repression of GUS activity was only observed in seed tissues on plants re-transformed with the seed specific effector construct NAP1-GAL4/HD. No repression of reporter gene activity was observed in leaf tissue in dual transgenic plants re-transformed with the seed specific effector construct (Figures 19 (A) and (B)).

These results demonstrate that sequentially transformed plants exhibit a similar repression of an expression construct (i.e. reporter gene) by an effector construct, as that observed in progeny produced from crosses between reporter plants (comprising an expression construct) and effector plants (comprising an effector construct).

Furthermore, these results show that repression of gene expression may be specifically targeted in a tissue-specific manner.

Example 6: Use of Plant Transcription Factors to Repress Developmental Pathway

Since the targeting of histone deacetylases to specific gene sequences using transcription factor DNA binding domains provides an effective method for repressing or silencing target genes. It was examined whether this approach is also useful for the repression of developmental, and metabolic pathways in plants. This example demonstrates that the plant transcriptonal factor Pti4 and histone deacetylase fusion proteins can be used to control plant developmental pathways.

Pti4 is a tomato transcription factor that belongs to the ERF (ethylene-responsive element binding factor) family of proteins. It interacts with the Pto kinase in tomato, which confers resistance to the *Pseudomonas syringae* pv *tomato* pathogen that causes bacterial speck disease. To study the function of Pti4, transgenic *Arabidopsis* plants were generated that expressed tomato *Pti4* driven by the strong constitutive promoters, CaMV 35S and -394tCUP.

Northern analysis (see below) demonstrate that expression of *Pti4* in transgenic *Arabidopsis* plants induced the expression of a GCC box-containing PR gene, basic chitinase, in *Arabidopsis*. It was also observed that Pti4 enhanced GCC box-mediated transcription of a reporter gene (see below). Expression of tomato *Pti4* in transgenic *Arabidopsis* plants produced a phenotype similar to that seen in plants treated with ethylene, suggesting that the *Pti4* gene is involved in the regulation of a subset of ethylene responsive genes which contain the GCC box. These results therefore suggest that tomato Pti4 acts as a transcriptional activator to regulate expression of GCC box-containing genes.

The Pti4 activator was therefore used as a controlling sequence-binding domain. Since Pti4 is capable of interacting with a controlling sequence, in this case a GCC box, the introduction of a *Pti4-AtHD2A* fussion into a plant should result in the repression of the phenotype associated with ethylene response. This in fact is observed, as the expression of the *Pti4-AtHD2A* fussion in transgenic *Arabidopsis*, represssed the ethylene-responsive phenotype.

Pti4 protein activates GCC box-mediated transcription of a reporter gene.

To test if the tomato Pti4 protein can interact with the GCC-box, *Pti4* effector plasmids were constructed in which the *Pti4* cDNA was driven by the strong constitutive promoters, CaMV 35S or tCUP (Figure 20 (A)). The reporter plasmids, GCC/GUS and mGCC/GUS (not shown), were constructed using a GUS reporter gene. Two GCC-boxes or mutated GCC-boxes (mGCC) (Ohme-Takagi and Shinshi, 1995) were fused to a minimal promoter, -62tCUP (Foster et al., 1999) to drive the GUS reporter gene expression. The effector plasmids were cobombarded into tobacco leaves together with a reporter plasmid. As shown in Figure 20 (B), co-transfection of the reporter plasmid GCC/GUS with a effector plasmid resulted in a 3 to 4-fold increase in GUS expression, indicating that Pti4 protein can interact with the GCC-boxes in the promoter of the reporter construct to activate transcription. Transcription of the reporter gene that had a mutated GCC-box was not activated by Pti4 (data not shown).

Ectopic expression of tomato Pti4 induces resident basic chitinase gene expression.

Transgenic *Arabidopsis* plants were generated that expressed *Pti4* driven by the strong constitutive promoters, CaMV 35S or tCUP (Foster et al., 1999). Southern blot analysis was performed to determine whether the genomic DNA of the putative transformants contained the transgenic DNA (data not shown). Four of the transgenic lines (tCUP/Pti4-1, tCUP/Pti4-3 and tCUP/Pti4-4, tCUP/Pti4-5) contained the *Pti4*

transgene driven by tCUP promoter and two transgenic lines (35S/Pti4-3 and 35S/Pti4-6) contained *Pti4* transgene driven by CaMV 35S promoter.

The expression of *Pti4* RNA in the transgenic lines was determined by Northern analysis. The predicted 1 kb transcript was detected in five transgenic lines, tCUP/Pti4-3, tCUP/Pti4-4, tCUP/Pti4-5, 35S/Pti4-1 and 35S/Pti4-2, using the *Pti4* cDNA probe. Low expression was also noted in tCUP/Pti4-3, and no expression was observed in wild-type plants (WT; Figure 21). One transgenic line, tCUP/Pti4-5, showed bands that were larger in size than the bands in the other lanes of the transgenic plants. This is most likely due to the downstream termination of transcription. Different levels of *Pti4* transcript accumulation were detected in the transgenic lines, with the transgenic line tCUP/Pti4-1 having the lowest level of *Pti4* expression.

Solano et al. (1998) reported that overexpression of another ERF (ethylene-responsive element binding factor) protein, ERF1, in transgenic *Arabidopsis* plants induced basic chitinase gene expression. Basic chitinase is an ethylene-responsive gene, which contains the GCC box in its promoter (Samac et al., 1990). Therefore, the expression of tomato *Pti4* in *Arabidopsis* was examined to determine if Pti4 could induce the expression of the *Arabidopsis* basic chitinase gene.

As shown in the Figure 21, the basic chitinase (BC) gene was expressed at a relative low level in the wild-type but was induced in the transgenic lines tCUP/Pti4-3, tCUP/Pti4-4, tCUP/Pti4-5, 35S/Pti4-1 and 35S/Pti4-2. The transgenic line tCUP/Pti4-1, which had the lowest level of *Pti4* expression among the 6 transgenic lines, did not show the induction of chitinase expression. The transgenic line tCUP/Pti4-3 that had the highest level of *Pti4* mRNA expression showed the highest level of basic chitinase mRNA accumulation. These data indicated that there was a general correlation between *Pti4* expression and chitinase RNA accumulation, suggesting that *Pti4* induced the expression of the basic chitinase gene in *Arabidopsis*.

Transgenic Pti4 plants display an ethylene-responsive phenotype

To evaluate the involvement of Pti4 in the ethylene signaling pathway, *Pti4* transgenic plant lines were examined for the ethylene-responsive phenotype. This phenotype is characterised by a triple response in *Arabidopsis* which includes inhibition of root and hypocotyl elongation, radial swelling of the hypocotyl and root, and exaggeration in the curvature of the apical hook (Ecker, 1995; Chang and Shockey, 1999). The hypocotyls of the etiolated transgenic seedlings were measured 72 hrs after germination. Among the 6 independent transgenic lines, four transgenic lines (tCUP/Pti4-3, tCUP/Pti4-4, tCUP/Pti4-5 and 35S/Pti4-1) with high *Pti4* gene expression showed inhibition of hypocotyl elongation, a phenotype similar to those observed in the constitutive ethylene response-mutants or in wild-type plants exposed to ethylene (Solano et al., 1998). As shown in the Figures 22 and 23, the seedlings from the transgenic line tCUP/Pti4-1 displayed strong inhibition of hypocotyl elongation. A similar response (inhibition of hypocotyl elongation) is observed in plants exposed to 1-aminocyclopropane-1-carboxylic acid (AAC), a precursor of ethylene biosynthesis (Figures 22, 23). The seedlings from the transgenic line tCUP/Pti4-1, which had a lower level of *Pti4* transgene expression, showed weak inhibition of hypocotyl elongation (Figure 22). These data indicated that there was a correlation between the *Pti4* expression and the inhibition of hypocotyl elongation.

Expression of Pti4-AtHD2A represses ethylene-responsive phenotype.

To test the effect of Pti4-AtHD2A protein on the ethylene signaling pathway, transgenic plants overexpressing *Pti4-AtFD2A* were generated by using tCUP promoter. The *Arabidopsis* transgenic plants expressing *Pti4-HD2A* fusion protein was examined for the ethylene-responsive phenotype. The hypocotyls of the etiolated transgenic seedlings were measured 72 hrs after germination. As shown in the Figures 24, wild type seedlings (Figure 24 (A)) exhibited hypocotyl elongation. Seedlings overexpressing Pti4 (Figure 24 (B) exhibited the ethylene

responsive phenotype (inhibition of hypocotyl elongation). However, seedlings from the transgenic line Pti4-HDA (Figure 24 (C)) did not display inhibition of hypocotyl elongation, demonstrating that Pti4-AtHDA fusion proteins repressed ethylene responsive phenotype in transgenic plants. These results indicate that the Pti4 functions as a controlling sequence-binding daomain and is capable of interacting with a controlling sequecne (GCC box), and target HD to repress gene expression of an endogenous gene in a targeted manner.

All citations are herein incorporated by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

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